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Neuroprotective roles and mechanisms of neuroglobin

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Abstract

Objective—The objectives of this work were to update and summarize recent experimental works on neuroglobin, mainly focus on its neuroprotective effects and the mechanisms.

Methods—The literature was reviewed using PubMed database, and some of the recent findings from our laboratory were included.

Results—Neuroglobin is a recently discovered tissue globin with a high affinity for oxygen and is widely and specifically expressed in neurons of vertebrate's central and peripheral nervous systems. Investigations in the past several years have advanced our knowledge on the functions and mechanisms of neuroglobin, but many issues remain unclear. Emerging reports have shown that overexpression of neuroglobin confers neuroprotection against neuronal hypoxia or ischemia-induced damage in cultured neurons and in cerebral ischemic animal models. Accumulating findings suggest several possible neuroprotective roles and mechanisms including ligand binding and oxygen sensing, modulation of cell signaling pathways and maintenance of mitochondria function.

Conclusion—Emerging experimental works suggest that neuroglobin is neuroprotective against hypoxic/ischemic insults, probably via ligand binding and oxygen sensing, modulation of cell signaling pathways and maintenance of mitochondria function.

Keywords

Neuroglobin; hypoxia; ischemia; neuroprotection; mechanism

INTRODUCTION

Globins are oxygen-binding proteins that are widely present in many taxa, including bacteria, plant, fungi and animal. Four members of globin family have been identified, including hemoglobin, myoglobin, cytoglobin and neuroglobin. Among them, hemoglobin and myoglobin are the best-known globin proteins. In 2000, neuroglobin was firstly identified as a new globin member that is expressed in brain neurons¹. Neuroglobin is a 151-amino acid protein in both mouse and human, sharing less than 25% identity with other vertebrate globins. Neuroglobin is also highly expressed in peripheral nervous system, endocrine tissues and retina². In the past 8 years, a large array of experimental works has investigated the functions and mechanisms of neuroglobin^{3–8}. In this short review, we will focus on the neuroprotective effect of neuroglobin against hypoxic/ischemic injury of neurons and the possible mechanisms.

TISSUE-SPECIFIC EXPRESSION OF NEUROGLOBIN GENE

The tissue-specific expression pattern of neuroglobin gene is most distinguishable for its physiological function. *In situ* hybridization showed that neuroglobin messenger RNA (mRNA) was widely distributed throughout the adult rat brain, including cerebral cortex, hippocampus and subcortical structures such as thalamus, hypothalamus, olfactory bulb and cerebellum^{9–11}. The distribution of neuroglobin protein is consistent with its mRNA localization, and the sub-cellular immunoreactivity is restricted to the cytoplasm.

Among all neuroglobin-expressing cells, the highest expression is seen in retina, with the estimated concentration about 100-fold higher than in the brain¹². Neuroglobin mRNA was detected in the perikarya of the nuclear and ganglion layers of the neuronal retina, whereas the protein was present mainly in the plexiform layers and in the ellipsoid region of photoreceptor inner segment¹³. The distribution of neuroglobin correlates with the subcellular localization of mitochondria and with the relative oxygen demands. These findings suggest that neuroglobin supplies oxygen to the retina, similar to myoglobin in the myocardium and the skeletal muscle. Although neuroglobin concentration in the brain is relatively lower than in the retina and considering neuron as the major cell type in the brain specifically expressing neuroglobin, neuroglobin could be a unique molecule that plays certain roles in maintaining normal neuron function and responding to pathological insults.

REGULATION AND INDUCTION OF NEUROGLOBIN GENE EXPRESSION

To date, the promoter sequence and transcription factors that regulate neuroglobin gene expression have not been well clarified. Analysis of the genomic sequence of human neuroglobin gene revealed a promoter region containing several putative Sp1-binding sites and at least three transcription starting points, but how neuroglobin gene is transcriptionally regulated remains to be defined¹. A common mechanism of neuron-specific gene expression is through the neuron-restrictive silencer element (NRSE) and its binding protein, the neuron-restrictive silencer factor (NRSF), the latter being highly expressed in non-neuronal cells, thereby inhibiting its NRSE-containing target genes in non-neuronal cells¹⁴. A strong NRSE motif has been found in the 5' upstream of human neuroglobin gene, and a weak one is found in the mouse neuroglobin gene, suggesting a possible regulation mechanism by neuron-restrictive silencer factor for the neuron-specific expression of neuroglobin gene¹⁵.

Neuroglobin mRNA and protein production were induced by hypoxia in cerebral neuronal culture¹⁶. However, it is controversial on neuroglobin expression in response to ischemia *in vivo*. For example, transient global forebrain ischemia up-regulated neuroglobin expression in gerbil cerebral cortex, but it was significantly down-regulated in the hippocampus after 20 minutes of bilateral common carotid artery occlusion followed by reperfusion¹⁷; in another work, neuroglobin expression was not altered in spontaneously hypertensive rats after transient middle cerebral artery occlusion (MCAO)¹⁸. The controversies are at least partially caused by the use of different antibodies and ribonucleotide probes^{2,9,18,19} and may be also due to the different physiological conditions of the animal models or the different testing time points.

Regarding the signaling pathways in the regulation of neuroglobin gene expression, one work suggested that the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase signaling pathway might be involved²⁰. In addition, hypoxia-inducible factor 1 (HIF-1) may also be one of the regulation factors¹⁶, which is supported by the consensus binding sequences (5'-RCGTG-3') of HIF-1 in the 5'-untranslated region of neuroglobin gene²¹. Moreover, neuroglobin expression can be induced by hemin, possibly through protein kinase G and soluble guanylate cyclase, indicating the involvement of different mechanisms in the regulation of neuroglobin gene expression²⁰.

NEUROPROTECTIVE ROLES OF NEUROGLOBIN AGAINST HYPOXIC, ISCHEMIC AND OXIDATIVE INJURY

The oxygen binding and neuron-specific expression properties make neuroglobin a new target molecule for the study of neuronal hypoxia/ischemia. Gene expression alteration approaches were applied to address whether neuroglobin is neuroprotective. The first report by Sun *et al.*¹⁶ showed that antisense-mediated knockdown of neuroglobin rendered cortical neurons more vulnerable to hypoxia, whereas overexpression of neuroglobin conferred protection of cultured neurons against hypoxia. Similar effect was observed in neuroblastoma cell line SH-SY5Y that neuroglobin over-expression enhanced cell survival under conditions of anoxia or oxygen and glucose deprivation²². In animal stroke models, intracerebral administration of a neuroglobin-overexpressing adeno-associated virus vector significantly reduced infarct size in rats after MCAO, and the outcome was reversed when neuroglobin antisense oligonucleotide was applied²³. Using neuroglobin-overexpressing transgenic (neuroglobin-Tg) mice, Khan *et al.*²⁴ found that the cerebral infarct size after MCAO was reduced by ~30% compared with wild type. Our laboratory also generated a neuroglobin-Tg mouse line and tested its neuroprotective effects in transient focal cerebral ischemia²⁵. Our results were broadly consistent with an earlier report from Dr Greenberg's group²⁴ and further documented that reduction of brain infarction in neuroglobin-Tg mice can be sustained up to 14 days after ischemia compared with wild-type controls, suggesting that neuroglobin overexpression is neuroprotective against transient focal cerebral ischemia, although the possible mechanisms need to be further characterized²⁵. We need to emphasize that the above experiments with non-tissue-specific neuroglobin transgenic approaches are 'outcomes' works. All findings are very informative for the effects of neuroglobin up-regulation in stroke but have limitations to fundamentally define or interpret the role of endogenous neuroglobin; therefore, a neuron-specific and inducible neuroglobin knockdown approach would be very useful in further investigation of neuroglobin function in normal versus ischemic brain.

MECHANISMS OF NEUROGLOBIN'S NEUROPROTECTIVE ROLES

Emerging experimental works suggest that neuroglobin is protective against hypoxic/ischemic brain injury^{24,25}. Although the underlying mechanisms remain poorly defined, initial evidences suggest that the neuroprotective mechanism of neuroglobin may be related to its structural features, putative signal transduction and maintenance of mitochondrial function.

Structural features and ligand binding properties

Neuroglobin protein in human or mouse exists as a monomer, which is distinct from the heterotetrameric hemoglobins. The three-dimensional structure of human²⁶ or mouse neuroglobin²⁷ has been solved, showing that the heme is inserted into the protein in two different orientations. The lack of orientation selectivity is possibly related to the presence of a large cavity lining the heme and to the increased mobility of heme contacts²⁷.

Spectroscopic and kinetic experiments indicate that human neuroglobin displays a typical globin fold, and the heme iron is hexacoordinate⁹, with proximal HisF8 and the distal HisE7 providing the two axial coordination bonds. Binding of exogenous ligand such as O₂ and CO displaces the endogenous HisE7 heme distal ligand. An elongated protein matrix cavity in the three-dimensional structure would facilitate O₂ diffusion to the heme²⁸. Neuroglobin was originally thought to function in O₂ storage and transportation similar to hemoglobin. However, the fact that neuroglobin exerts a high O₂ binding rate and low O₂ dissociation

rate, plus the relatively low neuroglobin protein concentration ($\sim 1 \mu\text{M}$) in the brain⁴, has cast doubt on these functions in general, although it may function in O_2 sensing^{29–31}.

A number of works have indicated that specific binding property of neuroglobin makes this molecule neuroprotective against hypoxic or ischemic injury via scavenging reactive species because neuroglobin has long been found to bind to NO directly with high intrinsic affinity and low dissociation rate³². In support of this function, a high degree of colocalization of neuronal nitric oxide synthase and neuroglobin has been detected within the anterior basomedial amygdala, lateral hypothalamus and laterodorsal tegmental nucleus³³, implying that in these neurons, NO could be the endogenous ligand for neuroglobin. Furthermore, Brunori *et al.*³⁴ found that the oxygenated derivative of neuroglobin, neuroglobin- O_2 , reacts with NO rapidly to produce NO_3^- and met-neuroglobin. This pathway would dispose of NO by means of a rapid reaction with neuroglobin- O_2 , which may in turn protect cellular respiration jeopardized by the inhibitory effect of NO on cytochrome c oxidase activity^{35,36}.

Dr Greenberg's group has extensively studied the effects of different oxidative challenges on cultured neurons with and without neuroglobin overexpression. Neuroglobin-transfected HN33 neuroblastoma cells were less sensitive to NO-induced cell death compared with wild-type cells, suggesting the ability of neuroglobin for neutralizing the neurotoxic effects of reactive nitrogen species³⁷. Neuroglobin overexpression conferred protection in SH-SY5Y cells directly injured by H_2O_2 ³⁸. β -Amyloid-induced cytotoxicity to PC12 cells, marked by reactive oxygen species production and lipids peroxidation, was ameliorated by neuroglobin overexpression³⁹. Findings from the above experiments suggest that neuroglobin may have the function of reactive oxygen species scavenging.

Regulation of signal transduction

In addition to the structural features and ligand binding properties described above, neuroglobin has also been hypothesized to act as a signal transducer. Dr Morishima's laboratory found that ferric human neuroglobin (met-neuroglobin) binds to the guanosine diphosphate-bound state of the α subunit of the heterotrimeric G protein ($G\alpha$) and exerts guanine-nucleotide dissociation inhibitor (GDI) activity⁴⁰. The ferric neuroglobin inhibits the exchange of guanosine diphosphate for guanosine triphosphate (GTP), thus preventing the $G\alpha$ subunit from binding to the $G\beta\gamma$ complex and activating the downstream signal transduction pathway, which is protective against oxidative stress⁴¹. In a continuous work, the binding sites between $G\alpha$ subunit and neuroglobin were identified⁴². This hypothesis was additionally supported by the observation that the GDI activity of human neuroglobin is required for its neuroprotection for PC12 cells under oxidative stress conditions. In this work, the human neuroglobin mutants that retained GDI activities rescued PC12 cell death caused by hypoxia/reoxygenation. In contrast, zebrafish neuroglobin and human neuroglobin mutants, which did not function as GDI, did not rescue cell death⁴³.

Khan *et al.*⁴⁴ recently showed evidence that neuroglobin binds two members of the Rho GTPase family, Rac1 and Rho A, as well as the Pak1 kinase, a key regulator of actin assembly and Rho-GDI-GTPase signaling complex activity under hypoxia. They thereafter hypothesized that neuroglobin may play a neuro-protective role by inhibiting the dissociation of the GTPase Rac-1 from its endogenous GDI, thus preventing the hypoxia-induced actin polymerization and microdomain aggregation. Moreover, neuroglobin was also found to be interacting with other targets such as flotillin 1 (a lipid raft microdomain-associated protein)⁴⁵ and the cysteine protease inhibitor cystatin C⁴⁶, suggesting a possibility that neuroglobin modulates the intracellular transport of cystatin C to protect against neuronal death caused by oxidative stress.

Based on these findings and together with the kinetics work of neuroglobin reaction with O₂ and NO, Brunori *et al.*³⁴ proposed that neuroglobin might function as a sensor of the relative O₂ and NO concentration in the tissue. Evidence presented that neuroglobin oxygenation is quickly reversible, and the oxygenated derivative, neuroglobin-O₂, reacts rapidly with NO to produce NO₃⁻ and met-neuroglobin. This process competes effectively with direct formation of neuroglobin-NO, which excludes the production of met-neuroglobin and its protective signaling function as a GDI⁴⁰.

The proper function of neuroglobin requires a met-neuroglobin reductase to maintain the balance between redox and oxidized neuroglobin. Complying with this scenario, Dr Brunori's laboratory recently opened another direction in exploring neuroglobin's functioning mechanism by finding that NADH/flavodoxin oxidoreductase (FIRd-red) from *Escherichia coli* is able to slowly reduce neuroglobin at catalytic concentrations⁴⁷. More interestingly, a BLAST search of the human genome showed that *E. coli* NADH/FIRd-red shares significant similarity with apoptosis-inducing factor (AIF), the principal mediator of the so-called caspase-independent programmed cell death⁴⁸. In healthy cells, AIF is located within the mitochondrion, but upon permeabilization of the mitochondrial outer membrane, it translocates first to the cytosol and then to the nucleus, where it triggers chromatin condensation followed by massive DNA fragmentation⁴⁸. It is therefore possible that AIF may reduce cytoplasmic met-neuroglobin on its way from the mitochondrion to the nucleus, and depending on O₂ tension, the reduced neuroglobin can either interfere with classical apoptotic pathway by reducing cytochrome *c* or bind O₂ and involve in NO scavenging³⁴.

Maintaining mitochondrial function

It has been well documented that neuroglobin expression is confined to metabolically active, oxygen-consuming cell types⁵. At the subcellular level, neuroglobin is associated with mitochondria and thus linked to the oxidative metabolism⁴⁹. It has been demonstrated that mitochondria comprises a central locus for energetic perturbations and oxidative stress in hypoxia/ischemia^{50,51}. Experimental works have shown that overexpression of neuroglobin promotes cell survival of PC12 cells against β -amyloid toxicity, attenuates β -amyloid-induced mitochondrial dysfunction³⁹ and eliminates hypoxia-induced mitochondrial aggregation and neuron death⁴⁴. Our laboratory has also demonstrated that neuroglobin overexpression improves mitochondrial function and reduces oxidative stress in neuroglobin-transgenic neurons after hypoxic insult in cultured mouse cortical neurons⁵². We found that at earlier time points after hypoxia/reoxygenation, no difference in neurotoxicity can be detected between neuroglobin-overexpressing and wild-type control neurons, whereas the rates of decline of several mitochondria functions biomarkers, including ATP levels, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide reduction and mitochondrial membrane potential, were significantly ameliorated in neuroglobin-transgenic neurons compared with wild-type neurons. Furthermore, neuroglobin overexpression reduced superoxide anion generation after hypoxia/reoxygenation, but glutathione levels were significantly improved compared with wild-type controls. Our data in this paper implicated that neuroglobin might affect both mitochondrial function and free radical generation as its potential neuroprotective mechanisms. However, there are multiple and probably inextricable feedback loops between preservation of mitochondrial energetics versus direct radical scavenging^{51,53–55}. We acknowledge that it will likely be impossible to unequivocally separate mitochondrial effects versus reactive oxygen species effects of neuroglobin.

To further dissect the molecular mechanisms of neuroglobin's neuroprotection, our laboratory recently searched for the interaction partners of mouse neuroglobin by performing yeast two-hybrid assay using a mouse brain complementary DNA library. We

identified several candidates that bind to neuroglobin, including Na/K ATPase β 1, cytochrome c1, ubiquitin C and ubiquitin-conjugating enzyme E2E1⁵⁶. Among these neuroglobin binding protein candidates, some of them are biologically important for neuronal function and survival. For example, cytochrome c1 is a subunit of the cytochrome bc1 complex (mitochondrial complex III) and is therefore of particular interest in explaining neuroglobin's function because cytochrome bc1 complex plays an important role in mitochondria function for energy transduction and generation of superoxide anion⁵⁶ and also in response to oxidative stress^{57,58} and regulation of HIF-1 activation induced by hypoxia^{59–61}. Cytochrome c1 is localized in the inter-membrane space between the outer and inner membrane of mitochondria⁶². The mitochondrial outer membrane contains the protein 'porin', which forms an aqueous channel through which proteins up to 10 kDa can pass and go into the intermembrane space. Hypoxia-induced superoxides as well as apoptotic agents such as Bax are able to cause permeabilization of mitochondrial outer membrane^{63,64}. As a 16-kDa monomer, neuroglobin might be able to pass the outer membrane to bind and affect the function of cytochrome c1. However, the phenomenon of neuroglobin binding to other proteins such as cytochrome c1 subunit and binding status-correlated alteration of associated cell signaling and mitochondrial function requires further investigation. The major hypotheses about neuroglobin-involved signaling pathways are briefly summarized in Figure 1.

SUMMARY

Neuroglobin is a newly discovered globin member, but its functions or roles in both physiological and pathological conditions remain poorly understood. Emerging experimental works suggest that neuroglobin is neuroprotective against hypoxic/ischemic neuron and brain injury, but the mechanisms have not been clearly defined. As an O₂-binding globin protein, the traditional O₂ storage and transport activity seems not realistic for neuroglobin. Instead, neuroglobin is more likely to act as a scavenger of reactive species, as well as a signal transduction molecule involved in neuroprotective pathways. Neuroglobin might also be closely related to mitochondria function. Based on the molecular property of neuroglobin and many works in cell free systems, some theoretical concepts have been proposed but have rarely been tested in cell cultures and animal models. Better understanding of the neuroprotective mechanisms of neuroglobin would advance our knowledge about the specific roles of neuroglobin in neurons or brain in response to hypoxia/ischemia for endogenous neuroprotection. Emerging data suggest that up-regulating or forcing neuroglobin expression has neuroprotective effects; thus, the strategy for elevation of endogenous neuroglobin expression may lead to a development of new approach for protecting against hypoxic/ischemic brain damage. Further investigations on the function and molecular mechanism of neuroglobin in both normal physiological and hypoxic/ischemic conditions are highly warranted.

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Figure 1.

A schematic description of possible functions of neuroglobin in signaling pathways involved in neuroprotection, including GDI activity that affects G protein-mediated signal transduction, influence on cytochrome *c* and AIF-mediated apoptosis, putative interaction with Rac-1 to affect actin polymerization and putative interaction with mitochondria cytochrome bc1 complex. Ngb, neuroglobin; GDI, guanine nucleotide-dissociation inhibitor; AIF, apoptosis-inducing factor; Cyt c, cytochrome c; Cyt bc1, cytochrome bc1 complex; ROS, reactive oxygen species